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Dr. Thomas E. Eurell

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Adult male rats have a strain, dose, and time-dependent renal proximal tubular degeneration induced by certain hydrocarbon compounds. We used rat strain variation (Fisher 344 and NCI Black Reiter) and different hydrocarbon compounds (JP-4, JP-8, decalin and trimethylpentane) to investigate the hydrocarbon-induced nephrotoxic response. Histochemical and morphometric evaluation of NCI-Black Reiter rats exposed to decalin and JP-8 indicated that this strain undergoes an intermediate form of the hydrocarbon-induced nephrotoxicity when compared to the albino Fisher 344 strain. The intermediate nephrotoxic response of the NCI-Black Reiter rat was characterized by approximately a two-fold increase in the number of acid phosphatase reactive lysosomes in renal tubular cells. The NBR rats did not demonstrate an increase in the size of the individual lysosomes, however, a characteristic lysosomal aggregation pattern occurred in renal tubular cells following hydrocarbon exposure. Light and electron microscopic immunohistochemistry revealed increased levels of A2U reactive sites in the renal tubular cells of F344 male rats exposed to decalin, trimethylpentane, JP-4 and JP-8. The nephrotoxic effect of decalin, trimethylpentane, JP-4 and JP-8 appeared to be equivalent as judged by renal tubular lysosomal alterations and increased A2U immunoreactive sites. Most lysosomal proteins in either control or treated animals were not reactive with specific antibodies against A2U. The relative number of A2U reactive sites per unit area did not increase as lysosomes enlarged or became angular in response to hydrocarbon exposure.

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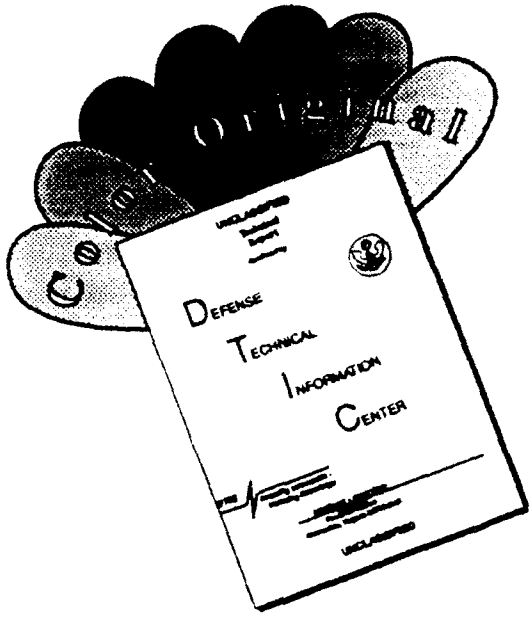


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# A COMPARATIVE STUDY REGARDING THE ASSOCIATION OF ALPHA-2U GLOBULIN WITH THE NEPHROTOXIC MECHANISM OF CERTAIN PETROLEUM-BASED AIR FORCE FUELS

AFOSR 90-0303

Final Technical Report (7/1/90-8/31/93)

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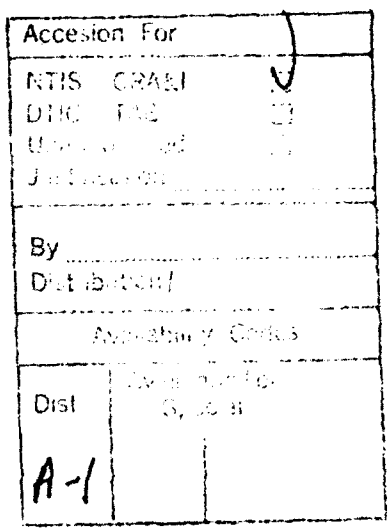


## SUMMARY

Adult male rats have a strain, dose, and time-dependent renal proximal tubular degeneration induced by certain hydrocarbon compounds. We used rat strain variation (Fisher 344 and NCI Black Reiter) and different hydrocarbon compounds (JP-4, JP-8, decalin and trimethylpentane) to investigate the hydrocarbon-induced nephrotoxic response. Histochemical and morphometric evaluation of NCI-Black Reiter rats exposed to decalin and JP-8 indicated that this strain undergoes an intermediate form of the hydrocarbon-induced nephrotoxicity when compared to the albino Fisher 344 strain. The intermediate nephrotoxic response of the NCI-Black Reiter rat was characterized by approximately a two-fold increase in the number of acid phosphatase reactive lysosomes in renal tubular cells. The NBR rats did not demonstrate an increase in the size of the individual lysosomes, however, a characteristic lysosomal aggregation pattern occurred in renal tubular cells following hydrocarbon exposure. Light and electron microscopic immunohistochemistry revealed increased levels of A2U reactive sites in the renal tubular cells of F344 male rats exposed to decalin, trimethylpentane, JP-4 and JP-8. The nephrotoxic effect of decalin, trimethylpentane, JP-4 and JP-8 appeared to be equivalent as judged by renal tubular lysosomal alterations and increased A2U immunoreactive sites. Most lysosomal proteins in either control or treated animals were not reactive with specific antibodies against A2U. The relative number of A2U reactive sites per unit area did not increase as lysosomes in renal tubular cells from treated animals enlarged or became angular in response to hydrocarbon exposure.


## INTRODUCTION

The exposure of Fisher 344 male rats to compounds used in this study results in a nephrotoxic response. The human renal response to hydrocarbon exposure is less clearly defined. Most human studies have focused on leaded gasoline and have failed to demonstrate any consistent association between long term, low level agent exposure and renal disease. However, the potential human health hazard of environmental or occupational hydrocarbon exposure has warranted continued epidemiological and mechanistic studies. Although the primary animal model for human risk assessment of hydrocarbon compounds is the rat, there is considerable controversy regarding the validity of this model. The basis for the controversy is centered on a urinary protein called alpha 2U globulin (A2U) which appears to be unique to the rat.

In animal studies of hydrocarbon exposure, toxic tubular epithelial cells characteristically have increased numbers of cytoplasmic protein droplets. The abnormal accumulation of protein droplets is termed hyaline droplet nephropathy and reflects the histologic sequelae of a dysfunctional protein catabolism following exposure to hydrocarbon compounds. The 2 major components of protein catabolism in the renal tubular epithelial cell are the endosomal pathway which brings the protein from the renal tubular lumen into the cell and the lysosomal pathway which is associated with post-endosomal processing of the protein. Hyaline droplet nephropathy is believed to be associated with inefficient renal tubular degradation of low molecular weight proteins (principally A2U).

The binding of hydrocarbon metabolites to A2U is hypothesized to render the molecule less susceptible to lysosomal digestion resulting in an excessive protein accumulation in the lysosome and eventual tubular epithelial cell death. The initial

hypothesis proposed that the lysosome enlarged to the degree that it eventually lysed causing cell necrosis and death. However, using lysosome-specific acid phosphatase staining and electron microscopic methods our research team demonstrated that the enlarged lysosomes were intact and that the process of tubular epithelial cell exfoliation and death more closely resembled apoptosis rather than necrosis. Although this study showed that the mechanism of cell death was not lysosomal lysis, the mechanism of cell exfoliation into the renal tubular lumen and eventual cell death following hydrocarbon exposure is still poorly understood. It is not known whether the toxicant action is due to a direct hydrocarbon effect on the tubular epithelial cell or to an indirect hydrocarbon effect associated with the binding of hydrocarbon metabolites to A2U.

Recent studies have suggested that the NCI-Black Reiter male rat may be less susceptible to the hydrocarbon-induced nephrotoxicity because this rat strain has relatively low levels of blood and urinary A2U. The principal investigator, in collaboration with toxicologists from the Armstrong Laboratory (AL/OET), Wright-Patterson AFB, is studying the association of A2U with the hydrocarbon-induced nephrotoxic process. The principal objectives of the study presented in this report were:

1. Use histochemical and morphometric techniques to evaluate the lysosomal alterations in Fisher 344 (A2U +) and NCI Black Reiter (A2U -) rats following exposure to JP-4, JP-8, decalin, and trimethylpentane.

2. Use immunohistochemical (light and electron microscopic) methods to assess the effect of JP-4, JP-8, decalin, and trimethylpentane on the presence of A2U globulin in tubular epithelial cells of the Fisher 344 male rat.

This final technical report represents our efforts from October 1, 1990 through August 31, 1993.

## ACCOMPLISHMENTS OF RESEARCH PROJECT

### Methods, Specific Reagents and Experimental Animals

1. Developed histochemical methods for acid phosphatase staining of lysosomes from experimental animals exposed to hydrocarbon compounds.

2. Developed specific immunologic reagents against urinary A2U from male rats.

3. Developed a combined histochemical/immunologic staining procedure for the simultaneous detection of renal tubular lysosomal alteration and A2U distribution.

4. Adapted a combined acid phosphatase/horse radish peroxidase staining procedure for the simultaneous detection of endosomal and lysosomal pathways in rat renal tubular cells.

5. NCI-Black Reiter (NBR) rats were obtained from the sole source in this country (National Cancer Institute) and a breeding colony was established at Wright-Patterson AFB.

Application of Methods and Specific Reagents

All rat exposures were conducted at the AL/OEVM facility of Wright-Patterson AFB. All animals were housed, maintained, and used in accordance with DHHS (NIH) Publication 85-23 entitled "Guide for Care and Use of Laboratory Animals," and the Animal Welfare Act of 1966, as amended. All procedures involving the use of animals were approved by the AAMRL institutional animal care and use committee prior to any experimental procedures. Hydrocarbon exposures were conducted on Fischer 344 (F344) and NBR rats according to a standardized protocol and tissues were collected on 5/23/91, 8/1/91, 9/13/91, 10/22/91, 12/17/91, 5/27/92, 11/20/92, 5/26/93, and 7/20/93. Tissues were recovered following gavage exposures to decalin (12 F344 + 12 NBR), trimethylpentane (12 F344 + 12 NBR), JP-4 (12 F344 + 12 NBR), and JP-8 (12 F344 + 12 NBR). Thirty-two control rats were also processed during the experiment. All tissues were processed by the principal investigator for immunohistochemical, histochemical and morphometric data.

Objective I-Histochemical and Morphometric Analysis

Histochemical and morphometric data revealed a distinct difference in the renal response of F344 and NBR male rats to hydrocarbon exposure. There was approximately a 2-fold increase in the number of lysosomes seen in renal tubular cells of treated NBR male rats. In addition, lysosomal aggregate formation occurred in NBR male rats exposed to hydrocarbon compounds. Control animals exposed to saline showed no change in the number or formation of renal tubular lysosomes. Representative sections of renal tubular cells stained with the lysosome-specific acid phosphatase stain are shown in Figure 1. Histochemical and morphometric data from Fisher 344 rats revealed the typical enlarged lysosomes with both decalin and JP-8 exposure (Figure 1). We presently believe that the NBR rat demonstrates an intermediate form of hydrocarbon-induced nephrotoxicity. Renal tissues processed using H&E, Mallory Heidenhain's, and Lee's Methylene Blue-Basic Fuchsin stains did not reveal this lysosomal alteration.

Objective 2-Immunohistochemical Analysis

**Light Immunohistochemistry**

A biotin-avidin linked, glucose oxidase system was used in conjunction with antibodies directed against A2U (pI 5.4 and 5.5 specific immunologic reagent) to detect the presence of A2U in rat renal tubular cells. Increased levels of A2U reactive sites occurred in the renal tubular cells of F344 male rats exposed to decalin, trimethylpentane, JP-4 and JP-8 when compared to controls. Representative kidney sections from control animals and rats exposed to decalin are shown in Figure 2. These sections were stained using a combined histochemical/ immunologic staining procedure for the simultaneous detection of renal tubular lysosomes and A2U. The nephrotoxic effect of trimethylpentane, JP-4 and JP-8 appeared to be equivalent to decalin as judged by renal tubular lysosomal alterations and increased A2U immunoreactive sites. The distribution of A2U immunoreactive sites in renal tubular cells from male F344 rats exposed to decalin, trimethylpentane, JP-4 and JP-8 was primarily within the apical portion of the proximal tubular cell, with only a limited number of A2U reactive sites detected in the enlarged lysosomes (Figure 2, Frame B).

**Electron Microscopic Immunohistochemistry**

A 2 step procedure was used to detect A2U reactive sites at the ultrastructural level in renal tubular cells. First, kidney sections prepared for electron microscopy were incubated with antibodies directed against A2U (pI 5.4 and 5.5 specific immunologic reagent). Second, the kidney sections containing adherent A2U antibody molecules were incubated with a Protein A-gold (10 nm diameter) probe. Increased numbers of A2U reactive sites occurred in the lysosomes of renal tubular cells of F344 male rats exposed to decalin, trimethylpentane, JP-4 and JP-8 when compared to controls (Figure 3). All 4 hydrocarbon compounds appeared to have an equivalent nephrotoxic effect as judged by ultrastructural lysosomal alterations and increased A2U immunoreactive sites. There were 2 distinct forms of enlarged lysosomes at the ultrastructural level, round lysosomes and angular lysosomes (Figure 3, Frame B). There were no appreciable differences in the relative number of A2U reactive sites per unit area between the small lysosomes, enlarged round lysosomes and the enlarged angular lysosomes seen in tissues from hydrocarbon exposed animals.

We were concerned that electron microscopic immunohistochemistry did not reveal A2U in the endosomal compartment of tissues from control and treated animals, whereas, A2U was clearly shown in the endosomal compartment using light immunohistochemistry. This may have been due to insufficient tissue fixation (osmium tetroxide was not used) or decreased antigenic reactivity of endosomal A2U associated with electron microscopy specimen preparation. The osmium tetroxide fixation step was omitted in processing the tissue for electron microscopic immunohistochemistry because the fixative interfered with the Protein-A gold reagent.

**Comparison of Light and Electron Microscopic Immunohistochemistry**

Hyaline droplet nephropathy is believed to be associated with inefficient renal tubular cell processing and degradation of low molecular weight proteins. Two features of hydrocarbon nephropathy, as it relates to the cellular processing of A2U, are clear in comparing the light and electron microscopic data. First, most lysosomal proteins in either control or treated animals were not reactive with specific antibodies against A2U. This was demonstrated by the number of A2U reactive sites which occurred at both the light and electron microscopic level. Second, as lysosomes in renal tubular cells from treated animals enlarged or became angular in response to hydrocarbon exposure, the relative number of A2U reactive sites per unit area did not increase (Figures 2 and 3). If the amount of A2U was critical to the development of enlarged or angular lysosomes the number of A2U reactive sites should have increased in proportion to increasingly severe lysosomal alterations. We believe these results suggest that A2U may not be a central element in the development of hyaline droplet nephropathy.


**Figure 1. Renal Tissue From Exposed And Control Male Rats Stained With Naphthol AS-TR Phosphate Pararosaniline.** Magnification (1000X), scale marking in Frame F=10 $\mu$m.

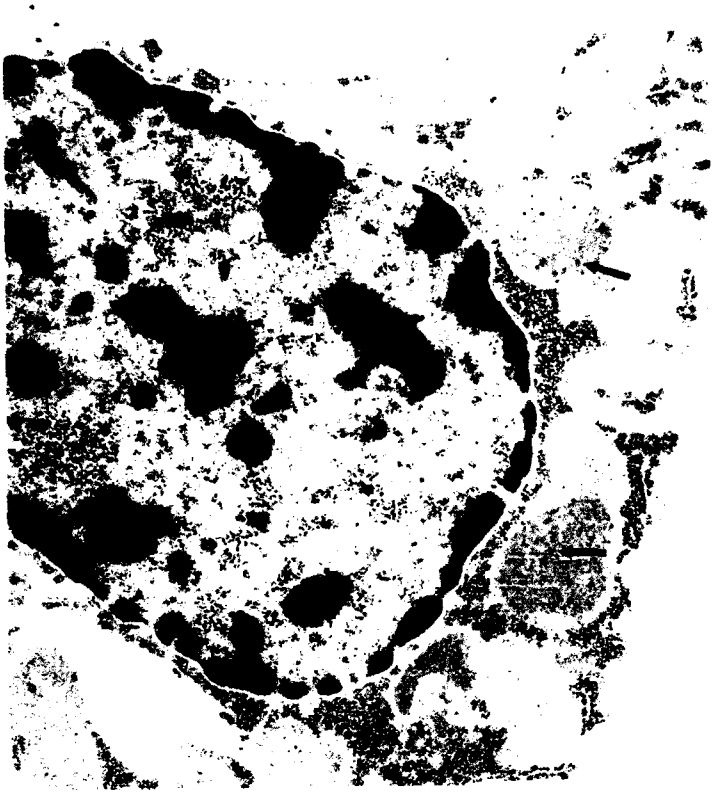
Frame A Control male F344 rat kidney section. The small cytoplasmic droplets (arrow) are due to the acid phosphatase reaction of the lysosomes.
Frame B Kidney section of male F344 male rat exposed to decalin. Note enlarged lysosomes (arrow) distributed along the basal border of the tubular epithelial cell.
Frame C Kidney section of male F344 male rat exposed to JP-8. Note enlarged lysosomes (arrow) distributed along the basal border of the tubular epithelial cell.

Frame D Control male NBR rat kidney section. The small cytoplasmic droplets (arrow) are due to the acid phosphatase reaction of the lysosomes.
Frame E Kidney section of male NBR male rat exposed to decalin. Note the increased number of lysosomes distributed along the median plane of the tubular epithelial cell. Arrow indicates a lysosome aggregate.
Frame F Kidney section of male NBR male rat exposed to JP-8. Note the increased number of lysosomes distributed along the median plane of the tubular epithelial cell. Arrow indicates a lysosome aggregate. Asterisk indicates an epithelial cell exfoliating into the renal tubular lumen.

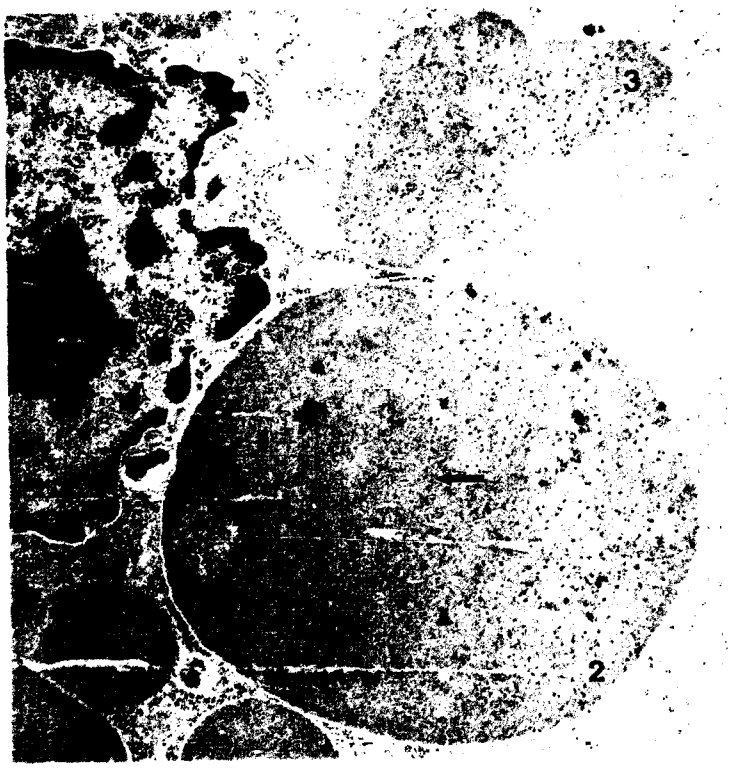
Frame A. Control Rat. A2U reactive sites (⟵) are located throughout the cytoplasm. Endolysosome formation is shown by combination of blue and red stains (⟵).

Frame B. Decalin Treated Rat. Note increase in A2U reactive sites (⟵) in cytoplasm, however, there is minimal endolysosome formation. A large angular lysosome is seen without any A2U reactive sites (⇦).

**Figure 2. Light Microscopic Immunohistochemistry Of Male F344 Rat Renal Tissue Following Decalin Exposure.** A2U reactive sites are stained blue and lysosomes are stained red. The kidney sections were counterstained with methyl green. Magnification (1000X)



Frame A. Control Rat. A2U reactive sites (⟵) are located within small lysosomes.




Frame B. Decalin Treated Rat. Note moderate increase in A2U reactive sites (⟵) in lysosomes. A small (1), large (2) and angular (3) lysosome can be seen in this frame. Note that the relative number of A2U reactive sites are similar in these different lysosome forms.

**Figure 3. Electron Microscopic Immunohistochemistry Of Male F344 Rat Renal Tissue Following Decalin Exposure.** A2U reactive sites are marked by 10 nm electron dense Protein-A gold probes. Magnification (15,000X)

Preliminary Evidence for a Non-A2U Based Mechanism of Hydrocarbon-Induced Hyaline Droplet Nephropathy

We adapted a histochemical technique used to evaluate cytoplasmic protein trafficking to the study of hyaline droplet nephropathy. The method involves:(1) intravenous injection of experimental animals with horseradish peroxidase 30 min prior to euthanasia, (2) preparation of kidneys for frozen sections and (3) double staining of the kidney sections with acid phosphatase (to stain lysosomes) and benzidine (to detect horseradish peroxidase). A normal distribution of horseradish peroxidase was seen within the endosomal and endolysosomal compartments of control renal tissue (Figure 4, Frame A). This agreed with previous reports using control animals and indicated that protein trafficking was occurring in a normal fashion. However, following decalin exposure: (1) the size and position of the lysosomes, (2) the position of the endosomes and (3) decreased endolysosomal formation within the renal tubular cells indicated a disruption in normal protein trafficking (Figure 4, Frame B). The effect of decalin exposure on protein trafficking was characterized by an accumulation of horseradish peroxidase in the endosomal fraction and a decreased formation of endolysosomes (e.g., very little of the endosomal material was released into the lysosome). This finding is directly analogous to the results of light microscopic immunohistochemistry, where the A2U reactive sites in kidneys from treated animals were principally located in the endosomal fraction (Figure 2).


**Figure 4. Horseradish Peroxidase/Acid Phosphatase Histochemistry Used To Detect Endosomal/Lysosomal Protein Trafficking.** (A) F344 Control Rat. Note normal size and perinuclear (median plane) location of lysosomes (red). The areas where endosomes (black) and lysosomes (red) combined to form endolysosomes are brown (arrow). This color change indicates a normal movement of protein from the endosomal compartment to the endolysosomal compartment. (B) F344 Rat Exposed to Decalin. Note enlarged size and basal location of lysosomes (red). There is a marked decrease in endosomal/lysosomal protein trafficking with only occasional endolysosome formation (arrow).

## SIGNIFICANCE OF RESEARCH FINDINGS

Comparison of the male rat renal response to hydrocarbon exposure suggests 2 histologic features which may be directly related to the mechanism of hyaline droplet nephropathy: (1) the cellular location of enlarged lysosomes in hydrocarbon-treated F344 male rats and (2) lysosomal aggregate formation in hydrocarbon-treated NBR male rats. The basal location of the enlarged lysosomes in the F344 rat following hydrocarbon exposure is unusual because lysosomes and endolysosomes are most often located at the median plane of the cell in the perinuclear cellular compartment (Figures 1 and 4). The NBR rat response to hydrocarbon exposure is unusual because of the lysosomal aggregates formed in the perinuclear cellular compartment (Figure 1, Frames E and F).

We propose that the unifying concept for the histologic evidence presented above and the mechanism of renal tubular cell exfoliation and death is a direct, hydrocarbon-induced alteration of cytoskeletal elements in the renal tubular epithelial cell.
Two general features of the cytoskeletal organization in epithelial cells support this hypothesis: (1) the maturation and movement of endosomes, endolysosomes, and lysosomes occurs along components of the cytoskeleton and (2) anchoring junctions between epithelial cells and the basal lamina (extracellular matrix) are joined to the cytoskeleton.

A hydrocarbon-induced alteration in the cytoskeleton could induce cross-trafficking of the endolysosome/lysosome complex and result in the aggregate formation seen in NBR rats (Figure 1). Results of a recent preliminary study conducted by our research team suggest that cytoskeletal alteration may be a generally applicable mechanism for hyaline droplet formation in both the albino and the pigmented rat strains. When exposed to decalin for an extended period (2 weeks) NBR rats had significantly enlarged lysosomes that would often be located in the basal aspect of the renal tubular epithelial cell in a manner similar to the characteristic F344 male rat response. Previous studies by Dr. David Mattie (AL/OET) using castrated and intact male F344 rats exposed to decalin for a short period (24 hr) showed groups of perinuclear lysosomal aggregates arranged in a manner similar to the characteristic NBR male rat response (Figure 5). Castration lessened the increase in renal tubular cell protein accumulation but it did not prevent protein accumulation nor did it prevent the enlargement and aggregation of the tubular cell lysosomes. These results are important to understanding the possible role of A2U in hydrocarbon-induced hyaline droplet nephropathy since normal A2U concentrations are dependent upon testosterone.

The anchoring junctions of the renal tubular epithelial cell (between adjacent epithelial cells and to the extracellular matrix) are joined to the cytoskeleton through adherens junctions, desmosomes, and hemidesmosomes. Alterations in the cytoskeleton can affect the adherens junctions causing detachment of the cell from the extracellular matrix. This is the mechanism of action for drugs such as cytochalasin which are used to disrupt cell-substrate attachments in tissue culture systems. Such a mechanism could explain the early exfoliation of proximal tubular epithelial cells seen following hydrocarbon exposure of male rats (Figure 1).

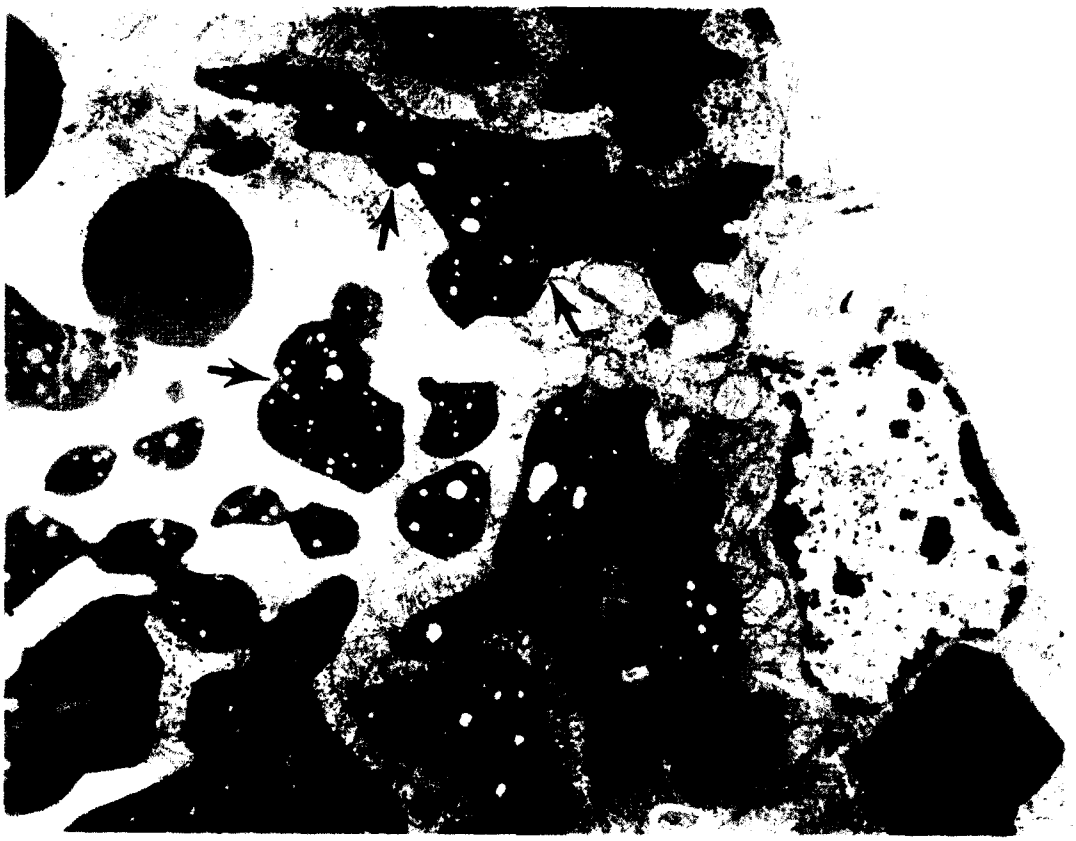


**Figure 5. Electron Micrograph Of F344 Male Rat Kidney 24 hr After Animal Exposure To 2 ml/kg of Trimethylpentane.** Photograph provided by Dr. David Mattie (AL/OET). Note areas (←) showing boundaries of individual angular lysosomes in aggregate formation.

## PUBLICATIONS

Published or Accepted for Publication

Davis, MA, and Eurell, TE. Evaluation of the association between alpha 2U globulin and lysosomes in male rats exposed to decalin. The Toxicologist 11(1):138, 1991.

Sinn, JM, Eurell, TE, Mattie, DR, and Alden, CL. Alteration of lysosomal morphology and endosomal marker distribution in renal cells of male rats exposed to decalin. The Toxicologist 11(1):137, 1991.

Eurell, TE, and Mattie, DR. Lysosomal alterations in F344 and NBR male rats following gavage exposure to decalin and JP-8. The Toxicologist 12(1): 428, 1992.

Eurell, TE, Mattie, DR, Dunovant, VS, Marit GB, and Flemming CD. Hematology and blood chemistry values for F344 and NBR male rats after exposure to decalin and JP-8. The Toxicologist 13(1): 205, 1993.

Eurell, TE, and Mattie, DR. The comparison of lysosomal alterations in Fisher 344 and NCI-Black Reiter male rats following gavage exposure to decalin and JP-8. Accepted by Toxicologic Pathology pending authors response to reviewer's comments.

Manuscripts In Preparation

Eurell, TE, Mattie, DR, Dunovant, VS, Marit GB, and Flemming CD. Hematology and blood chemistry values for F344 and NBR male rats after exposure to decalin and JP-8.

Davis, MA, and Eurell, TE. Evaluation of the association between alpha 2U globulin and lysosomes in male rats exposed to decalin.

Eurell, TE, and Mattie, DR. Alteration of lysosomal morphology and endosomal marker distribution in renal cells of male rats exposed to decalin.

## AWARDS

Davis, MA and Eurell, TE. Evaluation of the association between alpha 2U globulin and lysosomes in male rats exposed to decalin. Dr Davis received a Student Travel Award to the 1991 Annual Meeting of the Society of Toxicology. The competition for this award was based on the quality of the abstract submitted for the 1991 Meeting of the Society of Toxicology.

Davis, MA, and Eurell, TE. Evaluation of the association between alpha 2U globulin and lysosomes in male rats exposed to decalin. This poster received the 1991 Victor A. Drill Award for Best Poster Presentation at the Midwest Regional Meeting of the Society of Toxicology.

## GRADUATE STUDENTS

Myrtle A. Davis, D.V.M. received her M.S. degree in December 1991. The title of her thesis was "Immunochemical Localization of Alpha 2U Globulin in the Proximal Tubules of Male Rats Following Decalin Exposure".

## INTERACTIONS (COUPLING ACTIVITIES)

Dr. Eurell conferred with Dr. David Mattie (AL/OET) on 5/23/91. During that visit Dr. Eurell presented an update on this project, was involved in tissue collection, and planned the next set of experiments in collaboration with AAMRL staff.

Dr. Eurell conferred with Dr. David Mattie on 8/1/91, 9/13/91, 10/22/91, 12/17/91, 5/27/92, and 11/20/92. During those visits Dr. Eurell presented an update on this project and was involved in tissue collection.

Dr. Eurell conferred with Dr. David Mattie on 5/26/93. During that visit Dr. Eurell presented an update on this project and was involved in tissue collection. Dr. Eurell presented a seminar at AL/OET on 5/27/93 entitled "Biomarkers in Toxicology".

Dr. Eurell conferred with Dr. David Mattie on 7/20/93. During that visit Dr. Eurell presented an update on this project and was involved in tissue collection.